VERIFICATION OF A TRANSLATION

I, the below-named translator, hereby declare that:

My name and post office address are as stated below:

I am knowledgeable in the French and English languages, and that I believe the attached English translation of the French-language text entitled "PROCEDE DE DETECTION DE LESIONS DE L'ADN AU MOYEN DE COMPLEXES DE PROTEINES ET ELEMENTS PERMETTANT LA MISE EN OEUVRE DU PROCEDE", French Application No. 98/04064, filed March 26, 1998, is a true and complete translation of said text.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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PROCESS FOR THE DETECTION OF DAMAGE OF DNA BY MEANS OF COMPLEXES OF PROTEINS AND MATERIALS PERMITTING PRACTICING THE PROCESS

The present invention relates to a process for the detection of structural modifications of DNA by using recognition and/or repair proteins for the damage.

The invention also relates to material permitting the practice of said process.

In the following description, and in the claims, we define as:

- "damaging product", any specific pure chemical agent, any artificial mixture of chemical agents, or any natural composition of chemical agents or else any physical agent such as radiation, particularly ionizing and ultraviolet radiation, any biological agent such as a virus and exogenous proteins.
- "sensitized support": any support particularly a solid one having been treated with substances having a very high affinity for nucleic acids (DNA or RNA);
- "cellular extract": any partially purified cellular extract, natural protein or product from genetic engineering, purified or not.

It is known that DNA, the genetic information support, can be damaged by exogenous metabolic processes, such as akylation or oxidation of the bases, but more particularly by any exogenous genetoxic agent (xenobiological, physical and chemical agents) having apparent consequences as to the cellular viability if the damage is not repaired.

In addition to cellular death induced by these genotoxins, a process of mutagenesis can be induced or result from poor repair of the damage. The appearance of mutants is adapted to give rise to cellular disfunction and in particular to initiate tumoral develoment.

It is thus important to analyze and detect any structural modification of DNA which can be the origin of mutations. The detection of damage can relate to preparations of purified DNA and treated in vitro by a genotoxic agent but also cellular DNA from tissues removed by biopsy or whole organisms or cells cultured in vivo or ex vivo, after treatment with any genotoxic agent.

Thus, in the medication industry, there may be need to determine qualitatively and quantitatively either the genotoxic power, or the protective potential relative to a genotoxic effect, of a compound or a mixture of compounds. Moreover, it can be of interest to know the capabilities of repairing damage following a genotoxic treatment in a given

cell type. Such a determination is useful for cells during culture, or else isolated ex vivo. Applications of this type of determination are for example the detection of genotoxic xenobiologicals, the follow up of patients treated by chemotherapy, or else following up persons working in a medium polluted by genotoxic substances.

There are known various DNA tests, particularly in patent application EP 0 472 482, which relates to a dosage of microquantities of extracellular DNA present in a biological liquid, particularly in blood plasma.

There is known from French patent application No. 95 03230, a process for determining the presence of DNA damage from a repair signal. This process uses the step of reparative synthesis which takes place after any excision of damage present in DNA.

This synthesis, also called UDS (Unscheduled DNA Synthesis) has been used to detect in cells after incorporation of marked nucleotides in damaged and repaired DNA, the presence of damage in DNA, as well as the repair activity of the cells studied. This reparative synthesis has on the other hand been used in a test described by Wood et al. (Cell 1988, 53, 97-106), which used two purified plasmids, of different sizes, one damaged, the other undamaged, as a reference, incubated in the presence of a

purified cellular extract. In the presence of a predetermined concentration of dNTP, of ATP and of magnesium, the excision reaction of the damage has been reproduced in totality, proceeding from the recognition, passing through resynthesis incision-excision to ligation. After purification, and separation of the plasmid DNA on agarose gel, the radioactivity incorporated in the damaged plasmids and the controlled plasmids can be measured after autoradiography of the agarose gel. The percentage of damage thus repaired in vitro by these mechanisms is of the order of 5%.

The incision-excision activity can be directly measured by modification of the test described above according to a protocol published by the present applicants (Calsou and Salles, 1994, Biochem. Biophys. Res. Com., 202, 788-795 and Calsou and Salles, 1994, Nucleic. Acid Res., 22, 4937-4942). The process used to detect DNA damage from the reparative signal described in the mentioned French patent application No. 95 03230, uses the property of the extracts to carry out the repair steps, and permits detecting this damage from the signal obtained during the resynthesis step which follows excision. This process comprises the following different steps:

- preparing DNA by a method which consists:

- either in fixing the target DNA on a sensitized solid support, then subjecting this DNA to the action of at least one damaging agent,

- or subjecting the cells directly to the action of a damaging product, lysing the cells in a solution, then fixing the DNA on a sensitized solid support,

- subjecting this fixed damaged DNA to the reparative action of a cellular extract, this extracting comprising a marker,
- directly or indirectly developing the incorporation of this marker in the repaired DNA.

Avoiding any biochemical condition dependent on the synthesis of DNA, to improve the sensitivity of detection, to obtain other information on the repair system which deals with the studied damage, to enlarge the range of detectable DNA damage, there is used the capability of recognition and/or repair proteins, of interacting with any type of damage.

Thus, the test according to the present invention uses proteins capable of recognizing damage produced in vitro in purified DNA or else from cells isolated ex vivo, with increased sensitivity, the possibility of studying as a

first approximation the repair system used, a wider range of detector damage and also an important saving of time.

Thus, the quantitative and qualitative detection according to the present invention takes place in the first step of recognition, and not in the step of reparative synthesis. The conditions of interaction between the proteins and the damaged DNA can thus be optimized without taking account of the biochemical parameters required for all of the reparative reaction.

There is known at present at least about ten proteins adapted for this type of function within the scope of the excision of nucleotides, at least as to the basic excision, as well as other proteins implied in the recognition of breaking the DNA.

The process according to the present invention comprises the following steps:

- preparation of DNA,
- damaging treatment of this DNA, and
- fixation of this damaged DNA on a sensitized solid support,

or

- preparation of DNA,
- fixation of this undamaged DNA on a sensitized solid support, and

- damaging treatment of the DNA,

or

- treatment of cells,

- lysis and capture of the cellular DNA,

and is characterized in that it consists in:

- causing to act on this damaged DNA a composition comprising either a cellular extract having at least an activity for recognition and/or repair of the damage, or a purified protein with a known recognition spectrum, and

- detecting on the damaged DNA, directly or indirectly, the presence of the recognition and/or repair proteins for the damaged produced.

All the steps being separated by at least one step of washing if necessary.

More particularly, the process for qualitative and quantitative detection of damage, is characterized in that it consists in directly detecting in damaged DNA the repair proteins or any other recognition construction with the aid of antibodies or of marking systems and developing by chemoluminescence the formed complexes.

The antibodies comprise primary and secondary antibodies.

A first embodiment of the process consists in detecting the proteins connected to damaged DNA by a first specific antibody, followed by development of the ELISA type using a second antibody coupled to an enzymatic activity (for example HRP) permitting a quantification by luminescence.

There can also be detected in the supernatant the presence of repair proteins after separation on gel and amino blotting and/or studying the decrease of composition of these proteins as a function of an increased number of damaged DNA.

A variant usable for known damage consists in using directly a purified protein for specific repair and/or recognition and in detecting by the ELISA technique with the help of a primary antibody directed against the protein then a secondary antibody coupled to an enzymatic activity.

Preferably, the solid support is a microtitration plate with wells, or else any system using balls, so as to increase the capture surface of DNA, and the sensitivity of detection.

The solid support is sensitized by substances having a very high affinity for DNA, so as to promote a securement of this DNA by adsorption. These substances are selected

from cationic substances or proteins, at the pH used for the adsorption of nucleic material.

The cationic substances are selected from polyamino acids of the type of polylysine or polyarginine, levorotary, dextrorotary or racemic. In the case of polylysine, its molecular weight lies in the fraction 15,000 to 30,000 Daltons.

The sensitization of the support is carried out by incubation in a phosphate buffer of 10 mM, sodium chloride 137 mM and a pH comprised between 6.5 and 8, more particularly 7.

Preferably, the adsorbed DNA is genomic DNA obtained after lysis of the cells treated or untreated with a genotoxic agent.

The invention also covers the materials necessary for the practice of the process, which is to say:

- modified DNA,
- the cellular extract usable for all the activities of detection and/or repair of this damaged DNA,
- the incubation and washing buffers, and
- the microplate sensitized by adsorption of plasmid and cellular DNA.

Moreover, the materials can comprise lysis buffer for on the one hand the desorption of the complexes and on the

other hand the lysis of these cells when the detection is carried out on cellular DNA after damage.

Brief Description of the Drawings

The invention will now be described with respect to the accompanying drawings, in which:

- Figure 1 is a schematic view of steps of the process according to the principal embodiment, and a variation,
- Figure 2 is a view of the distribution ratios,
- Figure 3 shows amino reactivity of two repair proteins,
- Figure 4 shows a diagram of the repair ratio obtained with a purified cellular extract as a function of different types of damaging agents,
- Figure 5 shows a diagram of the results obtained by the process according to the invention,
- Figure 6 shows a diagram identical to that of Figure 5, but with a different protein,
- Figure 7 shows a diagram of the results obtained by the process according to the invention taking into consideration the detection of the DNA breakage, and
- Figure 8 shows a diagram of the results obtained by the process according to the invention taking into consideration the detection of the single strand breakage of DNA.

In Figure 1, the principal mode which comprises steps A, B, C and D1, corresponds to the direct detection by the ELISA technique of a protein complex for damage repair, with detection by chemoluminescence.

Variant D2 shown also in Figure 1, requires additional steps and is principally used to monitor the results obtained by the reaction D1. This variant corresponds to the series of the following steps: desorption of the recognition and/or repair proteins from the microplate, separation by PAGE SDS gel, transfer to nitrocellulose membranes, then detection by immunoblotting technique.

There is shown a step A which consists in fixing on the sensitized support DNA of purified plasmid origin and process to produce damage or else cellular DNA from cells treated by direct or indirect genotoxic agents, then lysed with a lysis buffer.

There can be cited an example of cellular lysis buffer called LB which comprises at least:

- 10 mM of phosphate buffer
- 10% of urea
- 1% of detergent
- 10 mM EDTA, pH8
- 100 µg/ml Rnase A
- distilled water.

The DNA is then immobilized directly and without purification on the support as is described in French patent application No. 95 03230.

The support can be a microtitration plate with wells or any other support using balls so as to increase the capture surface for the DNA and hence the sensitivity of detection in a reaction volume that is as small as possible.

The support is saturated for at least 15 minutes at 30°C with a saline phosphate buffer solution, PBST, to which is added 0.025% of acetylated bovine albumin serum.

In the course of step B, the support is used in a repair reaction which consists in incubation in the presence of purified cellular extracts, for two hours at 30°C to give an example, in the presence among others of a known composition of dNTP.

During the repair step, one of the nucleotides used is modified, for example biotin-21-dUTP, and the latter is incorporated in biotin-11-dUMP during the reparative synthesis step.

The incorporated quantity of modified dUMP is a function of the repair activity and can be detected by chemoluminescence, as has been described in French patent application No. 95 03230.

The object of step C is no longer to detect the presence of biotinylated dUMP with extravidine, but to determine on the same support the proteins present in damage which are specific to repair.

In the case of the study of reparative activity, there are obtained results as well as mentioned in Figure 2, which is to say the repair capacity of the DNA as a function of time, according to the quantity of damage produced by ultraviolet light.

Thus, Figure 2 shows a view of the ratio of distribution, which signal is obtained for damaged plasmid divided by the signal obtained for untreated control plasmid, this as a function on the one hand of the time of incubation of the reaction, and on the other hand of the quantity of damage produced in the DNA by ultraviolet light, and this for 3 different doses of irradiation.

The repair is conducted under normal conditions: temperature 30°C, 150 µg of purified cellular extract, 40 ng of treated or untreated plasmid, adsorbed on the support.

Figure 3 shows the immunoreactivity of two repair proteins which have been selected as references for the recognition of the ultraviolet damage of DNA:

- XPA, protein participating in the recognition of damage, and
- p62, protein of the transcription factor TFIIH, taking part in the pre-incision complex.

The presence of these proteins is followed with the help of specific antibodies on the treated or untreated plasmid, under reaction conditions in Figure 2, after two hours of incubation with the plasmids treated with 3 different doses of irradiation by ultraviolet light.

There will be seen the correlation between the amount of damage present by plasmids and the number of molecules of repair proteins taking part in the steps of the excision reaction of nucleotides.

According to the present invention, there is used a different approach from that of the repair synthesis.

Thus, the process according to the invention consists either in:

a) directly determining by immunodetection in the support, the quantity of repair and/or recognition proteins interacting specifically with the damage produced in the DNA, step D1 of Figure 1.

In this principal mode of determination, there is selected a specific primary antibody, and the latter is incubated with agitation. This antibody is for example

directed against the XPA protein, or else against the p62 protein, and is diluted in a solution of PBS and BSA according to the titer of the antibody used. The supports are then washed with a solution of PBST, then incubation is carried out of a secondary antibody conjugated for example with a peroxidase and diluted as a function of the antibody used. The supports are again washed. The development and quantification are carried by chemoluminescence.

or

b) determining the quantity of proteins after having carried out a desorption step in a suitable solution, and detecting by immunoblotting on nitrocellulose filter. This step D2 of Figure 1 serves to control the reaction D1 which is a process which is much easier and can be automated.

For example, the desorption of the repair proteins is carried out by using a DB buffer: 62.5 mM tris-HCI, pH 6.8, 4M urea, 10% glycerol, 2% SDS, 5% β-mercapto-ethanol, 0.003% bromphenol blue. This step lasts 30 minutes at about 30°C, with agitation. These proteins are then denatured by heating to 80°C to fix the order of size, for 20 minutes, and then agitation for 5 minutes at 30°C. More particularly, the detection by immunoblotting is carried out by SDS-PAGE electrophoresis, which permits

separating the proteins as a function of their size, and developing, after transfer onto membranes, the proteins of interest in the presence of specific antibodies. The obtained complexes are developed as above, by illuminescence.

Figure 4 shows a diagram of the ratio of the distribution obtained with a purified cellular extract, as a function of different types of damaging agents.

The modifications produced by UVC light and CDDP are recognized by the excision system of nucleotides, whilst those induced by MMS are recognized by the basic excision system.

No matter what the damaging agent, a repair ratio is obtained signifying the presence of damage in DNA, as well as has already been obtained by the quantification process of repair synthesis of the prior art.

In Figures 5 and 6, it is shown that the proteins tested belong to the nucleotide excision system, as XPA and -TFIIH-p62, the latter being recognized when the damage is recognized by this system, for example damage by UVC light or by CDDP, but are not recognized when the DNA is damaged by an agent which induces modifications not recognized by repair by excision of nucleotides, MMS for example.

These results indicate the specificity of the repair reaction, and there will be obtained an inverse image if there are used antibodies directed against specific glycoslyases of the alkylating agents, which will give a signal with the use of plasmids damaged by MMS for example, whereas they give no signal when the plasmid is damaged by UVC light or by CDDP.

In Figures 7 and 8, it is shown that there can be obtained proteins associated more specifically with double strand breakage or single strand breakage in DNA. Thus, a reactivity as to the complex Ku70/Ku80 indicates the presence of double strand breakages whilst reactivity relative to the poly-ribose polymerase protein, PARP, indicates the presence of single strand breakages in DNA.

In addition to the simplicity and sensitivity of detection of repair proteins, according to the antibody used, the repair system involved in the repair of unknown damage can be discriminated, and thus the conclusions of the study can be oriented toward the probable chemical nature of the damage.

Another approach consists in using, not extracts of cells having all the repair activities, but cells deficient in one of these systems, for example cells from patients

suffering from xeroderma pigmentosum, which are deficient in the initial stages of nucleotide excision.

For example, by using extracts from XPA cells, there is obtained no signal with XPA antibodies, because this protein is not produced by the cell, but there is also no signal obtained with TFIIH-p62 antibodies because it is necessary first to perform the step of recognition with XPA. Thus this step of recognition is necessary for the securement of the other proteins of the repair complex.

There can be carried out experiments with mutants of the basic excision systems, or with mutants of other proteins for repairing damage, such as single strand or double strand breakage of DNA.

Another approach consists in using a purified protein having a specific recognition spectrum for certain DNA damage.

The design of this test shows its extreme flexibility, and adaptability from the time at which antibodies of interest are available, the studies being adapted to be crossed with cellular extracts from mutants deficient in this or that repair activity, or with purified and/or recombinant proteins having an affinity for damage or a range of specific damage of DNA.

The invention also has for its object the combination of materials necessary to practice this process, these materials being preferably grouped in a container adapted for ease of commercialization.

Thus among the necessary materials, there are primary and secondary antibodies, the nature of these primary antibodies being adapted to vary according to the study in question. Thus in the case of basic materials, there can be supplied antibodies directed against a repair protein of the system of nucleotide excision, which system recognizes the quasi-totality of the modification of DNA.

In a more specific case, there are provided antibodies directed against certain proteins with a recognition spectrum that is narrower than that of the glycosylases or the recognition proteins for the breakage of DNA.